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Research article

The effect of the surface properties of poly(methyl methacrylate) on the attachment, adhesion and retention of fungal conidia

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Abstract: Poly(methyl methacrylate) (PMMA) surfaces, (commercial PMMA (PMMAc), spin coated PMMA (PMMAsc) and a 90% methylmethacrylate/10% 3-methacryloxypropyltrimethoxysilane random copolymer (P(MMA-co-gMPS))) were used to determine the effect of surface properties on conidia biofouling. The contact angles of the substrates demonstrated that the PMMAsc and the P(MMA-co-gMPS) polymer (62.8 °) were more wettable than the PMMAc surface (71.0 °). The PMMAsc had the greatest roughness value (32.0 nm) followed by the PMMAc (3.0 nm), then P(MMA-co-gMPS) (1 nm). *Aspergillus niger* 1957 conidia were spherical, smooth and hydrophobic (12.1%). *Aspergillus niger* 1988 conidia were spherical with spikes and hydrophobic (17.1%). *Aureobasidium pullulans* was elliptical with longitudinal ridges and hydrophilic (79.9%). Following attachment assays, cPMMA attached the greatest numbers of conidia. Following the adhesion and retention assays (washing step included in the protocol), *A. niger* 1957 and *A. niger* 1988 were least adhered to the P(MMA-co-gMPS) surface, whilst *A. pullulans* was least adhered to the PMMAsc surface. This work demonstrated that in the absence of a washing step, only the surface properties influenced the conidia attachment, whilst in the presence of a washing step, both the properties of the surfaces and the conidia affected conidia adhesion and retention. Hence, the methodology used (with or without a washing step) should reflect the environment in which the surface is to be applied.

Keywords: fungi; polymers; biodeterioration; attachment; adhesion; retention; spores; PMMA; *Aspergillus*; *Aureobasidium*

1. Introduction

Poly (methyl methacrylate) (PMMA), is a transparent thermoplastic. Due to its characteristic properties such as being lightweight and transparent, it is often used as an alternative to glass [1]. The increased knowledge in the properties of PMMA has contributed to the enhanced interest in PMMA synthesis, modification, and applications [2].

A major issue in the application of polymeric materials is fungal degradation [3]. Due to concerns over the use of biocides in the environment [4], it is important to understand how the interactions of the surface properties and microbial characteristics can influence biofouling, so that surfaces with anti-adhesive properties can be developed, thus potentially reducing their likelihood of biodeterioration. This biodegradation process typically begins with the attachment, adherence, and then the retention of the cells onto the surface [5]. Once fungal spores attach to a polymeric surface, hydration events may result in the release of an extracellular matrix and growth of the microorganism [6]. Over time, the fungi can degrade polymers *via* a number of mechanisms including physical disruption, enzymatic activity and increased reduction of the surface plasticizers. Such degradation can result in damaged, weakened and unsightly materials [7]. Previously, it has been determined that surface properties, such as the topography [8], chemistry [9,10] and physicochemistry [11] influenced initial cell or conidium attachment, adhesion and retention [12,13].

Aspergillus spp. produces fungal spores, which are dispersed into the air and can remain in the atmosphere for prolonged periods of time. The degradation of polymers has been shown to be related to the production of various organic acids produced by *Aspergillus* spp. [14]. Furthermore, the adhesion, germination and subsequent growth of *Aspergillus niger* conidia may cause surface deterioration on different substrates and has been associated with colonization of contact lenses [15]. It is the initial attachment and germination of the conidia that leads to the subsequent hyphal invasion [16].

Aureobasidium pullulans is a ubiquitous saprophytic fungus that is often described as an environmental contaminant in temperate, tropical and polar areas, as it has exceptional tolerance to a broad range of ecological conditions [17]. *A. pullans* has been shown to colonise a range of habitats including common household products, including bathroom surfaces [18], dishwashers [19], tap water [20] and the surfaces of synthetic polymers and polyvinyl chloride (PVC) plastics [7,21].

The fabrication method of the polymeric production will influence the substratum properties, this includes the surface topography and physicochemistry. Surfaces may be produced by manufacturing processes such as casting or spin coating, the latter allowing the chemical moieties of the substrata to be controlled. Often these properties are classified under the broader term of “responsive surfaces” [22].

An understanding of the interactions at the cell:substratum interface is essential to prevent biofouling [10]. The effect of the surface properties on the prevention of biofouling can be assessed by modifying surfaces or coatings with precisely controlled parameters. In order to better understand how different surface properties, affect the processes of spore attachment and retention to a range of polymeric surfaces, fungal conidia were used in a range of assays against three different surfaces, each with chemical variants of PMMA.

2. Materials and methods

2.1. Surface fabrication

Spin coated samples were prepared by spin coating a polymer solution or mixture onto a silicon wafer (Montco, USA). Two spin coated surfaces were produced and included PMMA spin coated from solution (PMMA_{sc}) and a 90% methylmethacrylate/10% 3-methacryloxy propyltrimethoxysilane (g-MPS) random copolymer P(MMA-co-gMPS). The desired polymer (or polymerising mixture) was dropped onto silicon wafer disks so that the entire disk was covered. Samples were centrifuged at 2000 rpm for 10–15 s.

2.1.1. Surface wettability

The sessile drop technique method using contact angle measurements (KRÜSS GMBH, Germany) at room temperature was carried out to determine the wettability of the surface using five microliter volumes of HPLC grade water (BDH, UK). Five different surfaces were used and three replicate contact angles were taken from each sample ($n = 5$).

2.1.2. R_a values of the surfaces

Roughness measurements were obtained using an Explorer AFM (Veeco Instruments, UK) in contact mode. The cantilever used had a spring constant of 0.05 N m^{-1} . In non-contact mode the cantilever spring constant was 50 N m^{-1} (Veeco Instruments, UK). Samples were carried out in triplicate on different surfaces ($n = 3$).

2.2. Mycology

2.2.1. Cultivation of fungi from lyophilizates

Fungal cultures were re-suspended from freeze dried lyophilizates. The fungal pellet was transferred into a test tube and 3 mL NaCl-Triton X-100 (*A. niger* spp.) or Sabouraud broth (*A. pullulans*) (BDH, UK) was added. NaCl-Triton X-100 was made by suspending one millilitre of a v/v 1% solution in 100 mL sterile distilled water, into which 0.85 g sodium chloride (Sigma, UK) was dissolved. The suspension was vortexed for 5 min or until the pellet was dispersed. The fungal suspension was dispensed onto Sabouraud agar plates (BDH, UK). The plates were incubated for 3 to 21 days at 29 °C. Following growth of the fungi, a second transfer was made. Inoculated plates were incubated for 3–21 days at 29 °C. After growth of the fungi across the plate, a third transfer was made. This transfer was used to obtain the “working” cultures from which conidia were harvested.

2.2.2. Conidia suspensions

Following fungal growth, 5 mL of a Sabouraud broth was pipetted onto the fungal culture. Conidia were removed from the culture by rubbing a sterile glass Pasteur pipette gently over the surface of the culture on the agar. The suspension was transferred into a sterile beaker containing a

sterile magnetic stirrer. This process was repeated several times until all the visible sporulating growth was removed from the agar. The suspension was stirred for 30 min then filtered through a funnel filled with glass wool (VWR, UK) to obtain a homogeneous conidium suspension. Conidia were harvested at 1721 g for 10 min, washed three times in sterile distilled water, and re-suspended to an optical density (OD) of 1.0 ± 0.1 at 610 nm which equated to $6.53 \pm 1.88 \times 10^6$ conidia cm^{-2} . Conidium suspensions were stored at 4 °C and used within two weeks and were checked using a light microscopy for purity prior to use.

2.2.3. Microbial adhesion to hexadecane

The hydrophobicity of the conidia was measured using an adapted microbial adhesion to hexadecane method [23]. PUM Buffer at pH 7.1 ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (BDH, UK) 22.2 g L^{-1} ; KH_2PO_4 (BDH, UK) 7.26 g L^{-1} ; Urea (Sigma, UK) 1.8 g L^{-1} ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (BDH, UK) 0.2 g L^{-1}) was used to wash the conidia thrice. The conidia were re-suspended in PUM buffer to an OD 1.0 ± 0.1 at 520 nm. The conidia in PUM buffer (5 mL) were added to glass, round bottomed test tubes and 1 mL of *n*-hexadecane (BDH, UK) was added. The suspensions were incubated for 10 min to equilibrate at 29 °C. The suspensions were mixed for 2 min, and incubated at 29 °C for 30 min. The lower aqueous phase was removed and transferred to clean test tubes and the OD was determined at 520 nm. Adhesion was calculated using the following equation as per Rosenberg et al. [23];

$$\text{adhesion} = \left(\frac{1 - A}{A_0} \right) \times 100$$

where *A* is the optical density following mixing with hydrocarbon and extraction of the aqueous phase and *A*₀ was the optical density of the spore suspension before mixing (*n* = 6).

2.2.4. Inoculation and preparation of conidia for Scanning Electron Microscopy (SEM)

Aliquots of 100 µL of conidium suspension was pipetted onto a 1 cm × 1 cm polished silicon wafer surface (Montco Silicon Technologies, US) and air dried in a class 2 microbiological containment hood. Surfaces were placed for 1 week in a desiccator containing phosphorous pentoxide (Sigma, UK). Substrata plus retained conidia were immersed in 4% v/v glutaraldehyde (Agar Ltd., UK) for 24 h at 4 °C. After fixing, substrata were washed gently with distilled water from a bottle at a 45 ° angle, with a 3 mm nozzle. Prior to examination, samples were stored at room temperature, in a desiccator containing phosphorous pentoxide. For SEM imaging, samples were fixed onto stubs for gold sputter coating (Polaron E5100, UK). Samples were coated at a vacuum of 0.09 mbar, for 3 min, at 2500 V, in argon gas at a power of 19 mA. Images of the substrata were obtained using a JEOL JSM 5600LV scanning electron microscope (*n* = 3).

2.2.5. Attachment, adhesion and retention assays

To carry out the attachment assay, three replicate substrata (1 cm × 1 cm) were attached to a stainless steel tray using adhesive gum (Impega, Malaysia). The tray and attached substrata were placed vertically in a class 2 flow hood. The conidium suspension (adjusted to an of 1.0 ± 0.1 at 610 nm) was placed into the spray reservoir of a Badger Airbrush (Shesto, UK). The airbrush was

propelled by a liquid gas canister (Esselte Letraset Ltd., UK). The airbrush was set to the finest spray setting, and at a distance of 10 cm the airbrush was passed left to right, ten times, at a speed of 50 mm sec^{-1} over the substrata. The flow rate was 0.2 mL sec^{-1} per pass. Immediately following spraying, the substrata were either laid horizontally and dried (attachment assay) or for the adhesion assay, the inoculated coupons were retained vertically and rinsed once, gently with 5 cm^3 distilled H_2O , with the distilled water bottle with a 3 mm nozzle at a 45° angle, to remove loosely attached conidia. Substrata with retained conidia were laid horizontally and air dried in a laminar flow hood. For the retention assay, three replicate substrata were placed horizontally in a sterile glass Petri dish. Twenty five milliliters of conidial suspension was added. The Petri dish containing the substrata was incubated without agitation for 1 h. The samples were removed, rinsed and air dried in a microbiological class 2 hood. The retained cells were stained for 2 min using 0.03% acridine orange in 2% glacial acetic acid (Sigma, UK), rinsed, and air dried. Substrata plus adherent conidia were visualised using epifluorescence microscopy (Nikon Eclipse E600, UK). The numbers of cells cm^2 was determined *via* a total of 60 fields of view across three different substrata, for each different surface type ($n = 3$).

2.3. Statistics

All results were presented as mean \pm standard deviation (SD). Mean values were compared using student t-tests to determine significant differences at the 95% confidence level ($p < 0.05$).

3. Results

The surface properties of the different PMMA substrata were determined in order to investigate their effects on conidia attachment, adhesion and retention.

3.1. Water contact angle measurement of substrates

The contact angles of the substrates demonstrated that there was no significant difference between the PMMA_{sc} and the P(MMA-co-gMPS) polymer (62.8°) whereas the PMMA_c surface was significantly different at 71.0° (Figure 1a).

3.2. Surface roughness values (R_a)

All the PMMA surfaces tested demonstrated low surface roughness and had R_a values within the nanoscale range (1.0–32 nm). The spin coated PMMA sample (PMMA_{sc}) had the greatest roughness value ($32.0 \pm 8.5 \text{ nm}$) followed by the PMMA_c sheet ($3.0 \pm 0.3 \text{ nm}$) and then the P(MMA-co-gMPS) ($1.0 \text{ nm} \pm 0.3 \text{ nm}$) (Figure 1b).

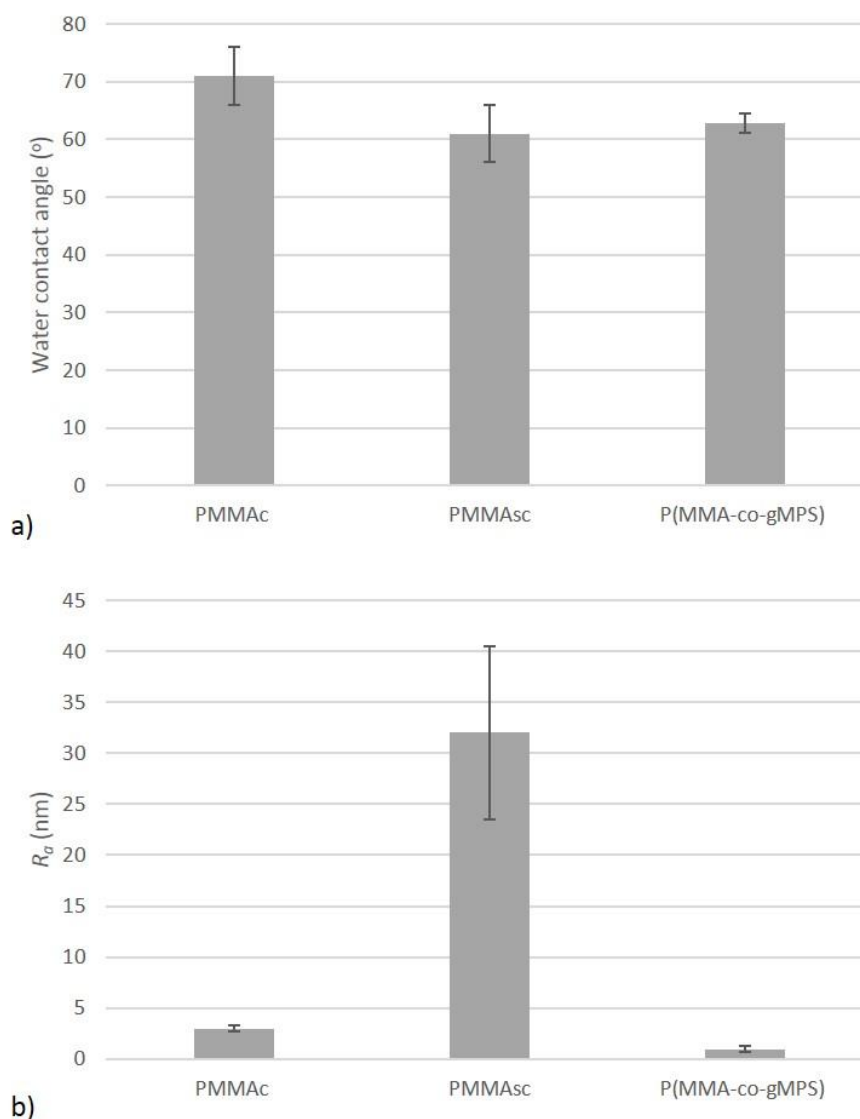


Figure 1. (a) Contact angle and (b) R_a values of the PMMA based surfaces.

SEM images were taken of the conidia (Figure 2), it was evident that the morphology of the conidia varied dramatically. *A. niger* 1957 conidia were spherical (overall diameter: 2 to 3 μm), with a smooth surface. *A. niger* 1988 conidia were also spherical (overall diameter: 4 to 5 μm) but with spike-like projections on its surface. *A. pullulans* was ellipsoidal in shape with longitudinal ridges. The presence of dried extracellular polysaccharide substances (EPS) was evident in the SEM images of *A. pullulans*.

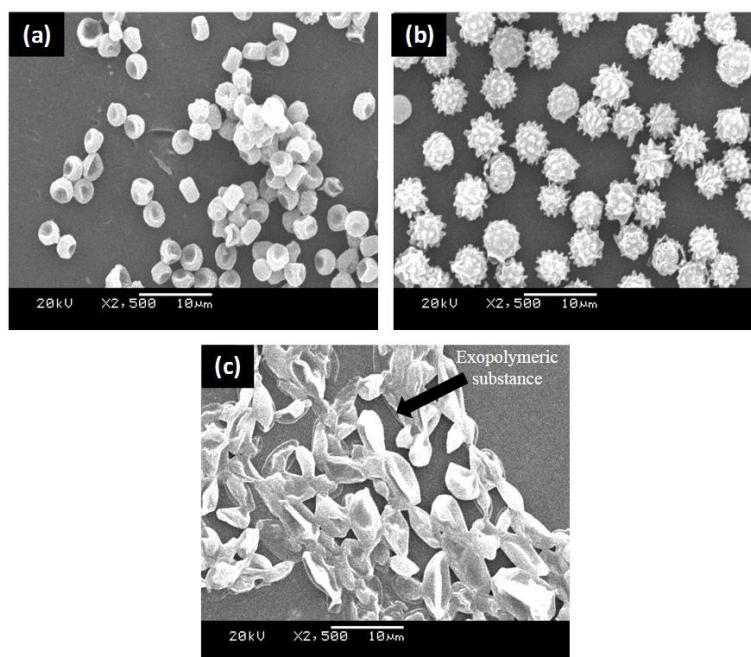


Figure 2. SEM images of desiccated fungal conidia: (a) *A. niger* 1957, (b) *A. niger* 1988 and (c) *A. pullulans*.

3.3. Affinity to hexadecane assay to determine fungal conidia hydrophobicity

Fungal conidia hydrophobicity was carried out using hexadecane and water (Figure 3). The absorbance in the aqueous phase for the three different conidia after treatment with both solvents demonstrated that *A. pullulans* was significantly different from the *A. niger* 1957 and 1988 spores since it was predominantly hydrophilic as it resided in the aqueous phase (79.9%) (Figure 3). *A. niger* 1957 (12.1%) and *A. niger* 1988 (17.1%) conidia were also significantly different from one another, however, they demonstrated hydrophobicity, with *A. niger* 1957 being the most hydrophobic.

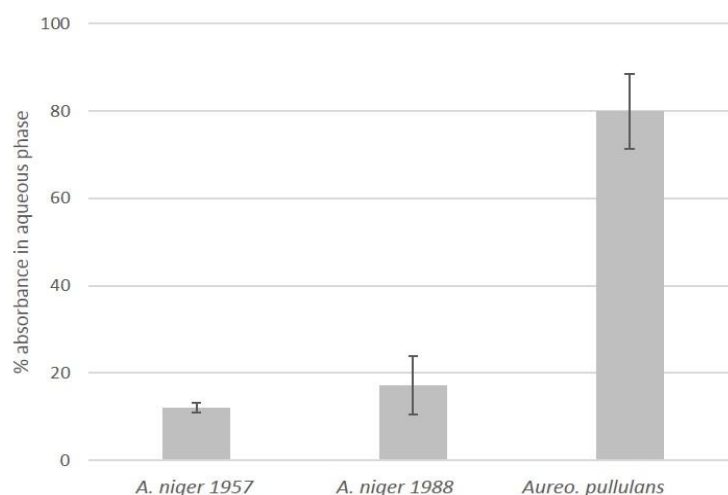


Figure 3. Affinity to hexadecane assay for the three fungal spore types.

3.4. Attachment, adhesion and retention assays

A range of microbiological assays were carried out to demonstrate how the conidia were retained on the surfaces under a range of conditions.

3.5. Attachment assay

Following the attachment assay, *A. niger* 1957 conidia demonstrated significantly greater levels of attachment to all three surfaces than the other conidia types, the greatest amount of conidia was retained on the PMMAc substrata (*A. niger* 1957: 3.88×10^5 conidia cm^{-2} , *A. niger* 1988: 7.62×10^4 conidia cm^{-2} and *A. pullulans*: 2.55×10^5 conidia cm^{-2}) whilst the PMMAc attached the least number of conidia overall (*A. niger* 1957: 9.34×10^4 conidia cm^{-2} , *A. niger* 1988: 2.08×10^4 conidia cm^{-2} and *A. pullulans*: 3.78×10^4 conidia cm^{-2}) (Figure 4).

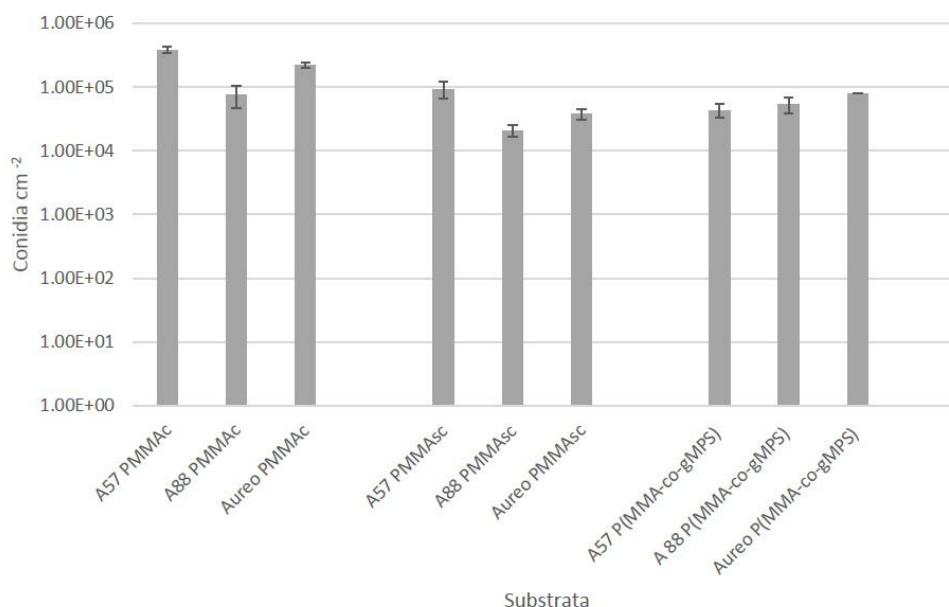


Figure 4. Interaction between the three fungal conidia and the substrata following the attachment assay (A57; *A. niger* 1957, A88; *A. niger* 1988, Aureo; *A. pullulans*).

3.6. Adhesion assay

For the *A. niger* 1957 conidia, the greatest numbers were adhered on the PMMAc and PMMAc surfaces (4.78×10^3 conidia cm^{-2} and 7.12×10^3 conidia cm^{-2}), respectively (Figure 5). The least number of conidia were adhered to the P(MMA-co-gMPS) surface (9.37×10^1 conidia cm^{-2}).

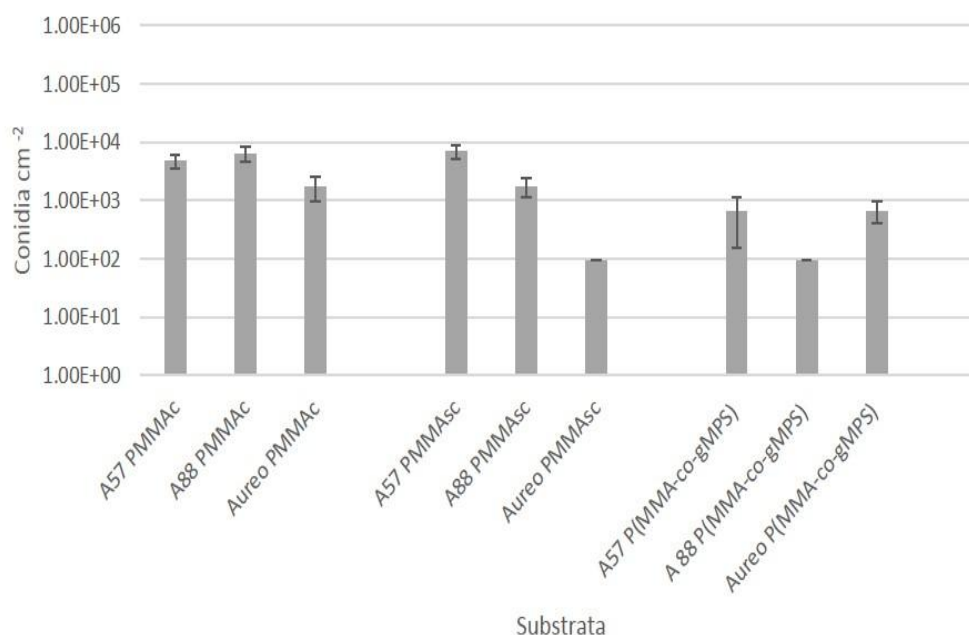


Figure 5. Interaction between the three fungal conidia and the substrata following the adhesion assay (A57; *A. niger* 1957, A88; *A. niger* 1988, Aureo; *A. pullulans*).

The PMMAc surface demonstrated the greatest adherence for the *A. niger* 1988 (6.46×10^4 conidia cm⁻²) compared with the PMMAsc (1.78×10^3 conidia cm⁻²) and P(MMA-co-gMPS) surfaces (9.37×10^1 conidia cm⁻²).

Following adherence assays with *A. pullulans*, there was a significant difference in the number of conidia adhered to all three surface types, with the greatest conidia amount observed on the PMMAc (1.78×10^3 conidia cm⁻²), followed by the P(MMA-co-gMPS) surface (6.72×10^2 conidia cm⁻²), and with the least amount of conidia on the surface, PMMAsc (9.37×10^1 conidia cm⁻²). Overall, *A. niger* 1957 and *A. niger* 1988 were least adhered to the P(MMA-co-gMPS) surface, whilst *A. pullulans* was least attached to the PMMAsc surface.

3.7. Retention assay

The PMMAc surface retained the most conidia, with all the fungal conidia being retained in similar numbers (*A. niger* 1957: 1.87×10^4 conidia cm⁻², *A. niger* 1988: 1.23×10^4 conidia cm⁻² and *A. pullulans*: 1.54×10^4 conidia cm⁻²) (Figure 6). Only the *A. niger* 1957 conidia on the PMMAsc retained similar numbers of spores (1.80×10^4 conidia cm⁻²). Whilst, the PMMAsc retained *A. niger* 1988 (3.37×10^3 conidia cm⁻²) and *A. pullulans* (1.03×10^3 conidia cm⁻²) at a similar level. The P(MMA-co-gMPS) retained *A. pullulans* conidia in the greatest numbers (2.81×10^3 conidia cm⁻²) followed by *A. niger* 1957 (9.37×10^2 conidia cm⁻²) and then *A. niger* 1988 (3.75×10^2 conidia cm⁻²). Thus, following the retention assays, *A. niger* 1957 and *A. niger* 1988 conidia were retained in the lowest numbers on the P(MMA-co-gMPS), whilst *A. pullulans* conidia was retained in the least numbers on the PMMAsc surface.

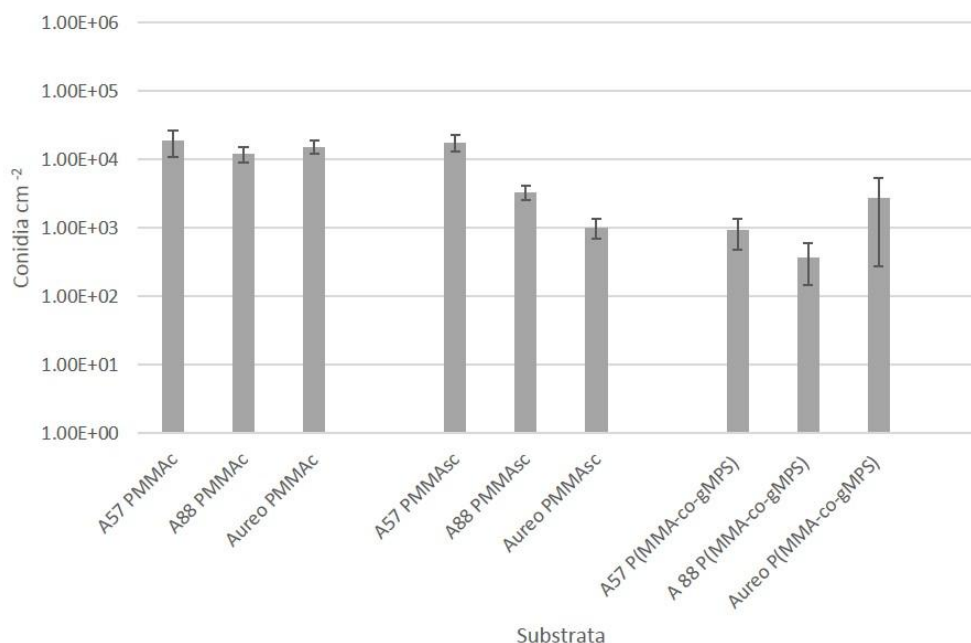


Figure 6. Interaction between the three fungal conidia and the investigated substrata following the retention assay (A57; *A. niger* 1957, A88; *A. niger* 1988, Aureo; *A. pullulans*).

4. Discussion

PMMA is utilised in a plethora of industrial applications. This study aimed to determine if the differences in the surface properties of a range of PMMA based substrata influenced fungal conidia attachment, adhesion and retention. Contact angles using water were reported from the PMMA derivatives and all surfaces resulted in values between 0° and 90°, which demonstrated wettable substrata. The contact angle values of the c-PMMA, P(MMA-co-gMPS) and PMMAsc represented moderate hydrophilic attributes as would be expected from the structure of such polymers [24].

In order to determine the surface topography of the PMMA surfaces R_a values were reported. Although a difference within an order of 10 was observed, all the surfaces had R_a values of less than 33 nm. The lowest R_a value was demonstrated on the PMMAsc and was closely followed by the PMMAc substrata. During the fabrication process of PMMAc, the sheet is cast against glass sheets, therefore it was not surprising that the R_a value was low and in agreement with our results, PMMAsc has previously been shown to have a R_a value of <5 nm [25]. The increased roughness observed from the spin coated surface, P(MMA-co-gMPS) may be due to the more widely distributed chemical structures of the surfaces producing a greater R_a value [26].

It is important to understand the fundamental mechanisms that influence the binding of fungal spores to surfaces. This requires enhanced knowledge of the interactions between the conidia and the physicochemical, chemical and roughness properties of the surfaces. With regards to the surface physicochemistry affecting fungal spore binding to a surface, it has been shown that fungal spores may be strongly adhered to hydrophilic substrates [27]. In agreement with our study, previous work has demonstrated that pycnidiospores [28], *Magnaporthe grisea* conidia [29], and the conidia of

Penicillium expansum [30] were found to be significantly more adherent to a non wettable surface. However, other studies have demonstrated that surface wettability did not play a major role in the adhesion of conidia [31].

Surface topography has been shown to influence the number bacterial spores that adhere to a surface [32]. However, Foschino et al. [33] demonstrated that the type of stainless steel finish (shot treated or not) had no significant effect on the cleanability of *A. niger* from the stainless steel. The results presented in this work demonstrated that the surface properties, including roughness did affect fungal conidia binding to the surfaces, but the results were dependant in part on the methodology used.

The affinity to solvent assay demonstrated a significant difference in the surface physicochemistry of the conidia hydrophobicity. Previous literature has shown that fungal conidia are highly hydrophobic [34]. The cell wall of *A. niger* is highly dynamic and contains several classes of polysaccharides, including, β -glucans, α -glucans, chitin, galactomannan, and cell wall mannoproteins [35,36]. The cell morphology and therefore the conidium shape may therefore influence both attachment and adhesion. The spiny ornamentation may reduce the contact area of the conidia with smooth surfaces but may also increase the conidia numbers by interlocking on dynamic or responsive surfaces. The solvent assay confirmed that *A. pullulans* produced the most hydrophilic conidia. This is possibly due to the production of the polysaccharide, pullulan, as it has been suggested that it can form a hydrophilic, non-ionic surface layer that can reduce protein adhesion [37].

Following attachment, adhesion and retention assays it was demonstrated that the attachment assays generally retained most conidia whilst the adhesion assays resulted in the least attached conidia. This could be due to the conidia being unable to bind to the surface quickly enough, prior to the surface being washed.

Following the adhesion assays, *A. niger* 1957 conidia were generally retained in greater numbers than the *A. niger* 1988 conidia on the spin coated surfaces. This may be due to the varying spore morphology, as *A. niger* 1957 exhibited a rounder structure which can increase conidial attachment. The rougher morphology of the *A. niger* 1988 conidia, demonstrated a higher hydrophilicity and a spiny outer coat, the overall result being a lower surface contact and the lowest levels of adhesion/coverage observed.

Overall, this work demonstrated that the method of assay used is of paramount importance. Although it is possible to modify PMMA surfaces in order to control biodeterioration [38], this work aimed to look at PMMA surfaces that were chemically and structurally similar. In assays without a wash step, only the surface properties were found to influence conidia attachment, adhesion and retention; although it should be noted that all the surfaces were comparably smooth (1–32 nm). Following the assays, it was demonstrated that generally all three conidial types adhered in the greatest numbers to the PMMAc surfaces (which was the most hydrophobic and least rough surface), whilst all the three types of conidia were attached in the least numbers to the PMMAsc (the most hydrophilic and roughest surface). This may also be in part due to the chemistry of the surfaces. The PMMAsc is PMMA spin coated from solution (in tetrahydrofuran (THF)) onto a silicon wafer. The way in which this surface was fabricated could have resulted in branched chains of the polymer, which may have made the surface more dynamic which could potentially reduce conidia binding to the surfaces. In the attachment assay, the differences in the size, shape and chemistry of the conidia had no influence on microbial attachment, rather the results were driven by the surface properties.

In assays with a washing step (adherence and retention) the surface and the conidia properties influenced the results. Although as with the attachment assays, the PMMAc retained the most conidia, regardless of the species. Following the adhesion and retention assays, the *A. niger* spores were the least retained on the P(MMA-co-gMPS) surface, whilst the *A. pullulans* were least retained on the PMMAsc surface. This effect may be due to the rougher PMMAsc surface topography demonstrating a greater contact area between the conidia and the surface. This is in agreement with the findings of Nomura et al. [27] who demonstrated that the area between a spore and substrate depended on the substrate roughness. The results demonstrated throughout the different assays could potentially explain the variance in opinions regarding the influence of surface and microbial properties observed in the literature.

5. Conclusion

This work used three chemically similar polymers with different surface roughness and wettabilities to determine if the effect of the surface properties could be related to the amount of conidia attached. Three different types of binding assays, attachment, adhesion and retention were utilised. It was demonstrated that the least wettable surface generally retained the most conidia. This work further demonstrated that the method of assay used to assess conidia attachment is of paramount importance. In the attachment assays, only the surface properties were found to influence conidia retained. In assays with a washing step (adherence and retention) the properties of the surface and the conidia influenced the results observed. Thus, the assay used to assess the surface efficacy should reflect the microbial:surface interface of the applied environment.

Author contributions

Kathryn A. Whitehead and Christopher M. Liauw carried out the laboratory work. Kathryn A. Whitehead produced the first draft of the work and oversaw subsequent manuscript preparation. Joels S. T. Wilson-Nieuwenhuis and Anthony J. Slate were involved with manuscript preparation and writing. Ted Deisenroth and Andrea Preuss designed the conceptualisation of the project and funded the work. Joanna Verran oversaw the project.

Conflicts of interest

The authors report no conflicts of interest.

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